

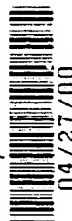


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Patentanmeldung Nr. Patent application No. Demande de brevet n°

99107397.4

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

Le Président de l'Office européen des brevets
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I.L.C. HATTEN-HECKMAN

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Sheet 2 of the certificate
Page 2 de l'attestation

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Anmelder:
Applicant(s):
Demandeur(s):
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DNA-construct for the tissue specific expression of a blood coagulation factor

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CENTEON PHARMA GMBH
ANR 8177007**1999/Z004 – Ma 1204 – C 36**
Dr. Lp / vm**DNA-construct for the tissue-specific expression of a blood coagulation factor**

Subject of the invention is a DNA-construct for the tissue-specific expression of a blood coagulation factor.

It is known that Factor IX (FIX) is a vitamin-K dependant glycoprotein which is produced by hepatocytes and secreted into the blood circulation (Kurachi et al., 1995; Salier et al., 1990). The FIX gene is located on the X-chromosome and contains 8 exons and 7 introns (Camerino et al., 1984; Boyd et al., 1984; Yoshitake et al., 1985). Absence or deficiency of Factor IX causes a severe bleeding disorder called hemophilia B (Roberts et al., 1993).

Hemophilia B is a disorder arising from modifications of a single known gene. It was therefore early proposed as a potential target for gene therapy. The cells chosen as target have to be easily accessible for gene transfer and need to be able to express Factor IX at a significant level. In these target cells, the FIX molecule needs to be modified to become an active coagulation factor (γ -carboxylation and cleavage of the propeptide). Several potential target cells were tested such as hepatic cells (Nakai et al., 1998), keratinocytes (White et al., 1998), muscle cells (Baru et al., 1995; Wang et al., 1997), or bone marrow stromal cells (Cherington et al., 1998). In vitro, the human myeloid leukemia cell line (HL60) transfected with a retroviral vector bearing a human FIX cDNA was shown to be able to produce a biologically active FIX (Hao et al., 1995). This invention is directed to the expression of blood coagulation factors in hematopoietic cells and specifically in platelets.

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As hematopoietic cells the human erythroleukemia cell line HEL has already been used which mainly expresses an erythroid phenotype (Martin et al., 1982) but also some megakaryocytic markers like platelets membrane glycoproteins (Tabilio et al., 1984). After induction by phorbol-12-myristate-13-acetate (PMA), HEL cell line expresses increased amounts of megakaryocytic proteins like glycoprotein IIb/IIIa complex, Platelet Factor 4, or von Willebrand Factor (Long and coll., 1990). The PMA effects are mediated via the activation of protein kinase C (Nishizuka et al., 1984; Hong et al., 1996).

The human platelet glycoprotein IIb (GPIIb) is the α subunit of the platelet integrin α IIb β 3 also known as IIb-IIIa complex which functions as a specific receptor for fibrinogen, fibronectin and von Willebrand's factor (Haas and Plow, 1994). GPIIb cDNA was initially isolated from mRNA libraries from HEL cells and human megakaryocytes (Uzan et al., 1988; Frachet et al., 1990). The human GPIIb gene was identified by Prandini et al. (1988).

The tissue specificity of the GPIIb promoter was assessed using the chloramphenicol acetyltransferase (CAT) reporter gene. Under GPIIb promoter control, a CAT expression was only detected in the hematopoietic cell line HEL (Uzan et al., 1991).

In order to treat hemophilia B by gene therapy it is necessary to have a tissue-specific expression of Factor IX. The present invention provides a solution to the problem of expressing blood coagulation factors in such hematopoietic cells.

It has now been found that a DNA-construct is suitable for a tissue-specific expression of a blood coagulation factor if it comprises a DNA coding for an amino acid sequence of a blood coagulation factor and a DNA coding for a promoter which is specific for the expression in hematopoietic cells. The solution to the above-men-

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tioned problem has been demonstrated with a DNA-construct wherein as promoter the DNA coding for the human platelet glycoprotein IIb (=GPIIb) has been used.

The development of said DNA-construct was performed as follows:

A. Construction of the chimeric molecules

1. Generation of the FIX cDNAs:

Wild type FIX gene contains 3 ATG codons in frame clustered at aminoacid -46, -41 and -39. In order to try to improve FIX production, five FIX cDNA expression vectors with different ATG sites have been produced, directed by the CMV promoter. The truncated FIX intron 1, previously shown to have an expression-increasing activity in HepG2 cell line (Kurachi et al., 1995) was cloned and introduced in FIX cDNAs. In an initial part of this project, stable transfections of CHO cells with CMV-FIX(-41S) and CMV-FIX(-41S.I1) constructs led to the best FIX production in the culture supernatants. These two constructs FIX(-41S) and FIX(-41S.I1) cDNAs which are bearing only the -41 ATG were therefore the first FIX constructs used to transfect the hematopoietic cell line. The modified 5'extremity of the FIX(-41S)cDNA is

-41S: 5'TGGATCCTTGGAGCGCGTGAACATGATCTTGG 3'
 -46 -41 -39

However, all the results obtained with FIX(-41S) cDNAs were further confirmed with the FIX(WT) and FIX(WT.I1) cDNAs. The 5'extremity of FIX WT cDNA is

WT: 5'TGGATCCATGCAGCGCGTGAACATGATCATGGCAGAA 3'
 -46 -41 -39

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2. Generation of the GPIIb promoter vectors

The lineage-specific promoter GPIIb was chosen to express FIX transgenes in hematopoietic cells. The pBLCAT-GPIIb vector bearing the -643/+33 GPIIb promoter and the chloramphenicol acetyltransferase (CAT) reporter gene was obtained from G. Uzan.

In order to use pcDNA3 vectors (Invitrogen, the Netherlands) as an expression vector for FIX (which has more restriction enzyme sites and a neomycin resistance gene and appeared, therefore, more convenient for the next studies), the endogenous CMV promoter was replaced by the GPIIb promoter. This promoter was sorted from the pBLCAT-GPIIb vector after HindIII-BamHI digestion. This product encompassed -597 to +33 fragment of the GPIIb promoter (see Sequence Listing, Annex 1) comprising the crucial domains for the promoter specificity (Uzan et al., 1991 and 1995). It was introduced in a CMV-deleted pcDNA3 opened by the same enzymes. The construct obtained was the so called pcDNA3-GPIIb (Figure 1). All the FIX cDNAs were sub-cloned in pcDNA3-GPIIb plasmid using BamHI-XhoI digestion.

3. Generation of the β -Galactosidase vectors

To test the activity of the truncated (-597/+33) GPIIb promoter in HEL cell line, the β -Galactosidase reporter gene was chosen. The pUT535 vector (Cayla, Toulouse, France) where the β -Gal gene expression is directed by the CMV promoter and enhancer (Figure 2) was chosen. To exchange the CMV sequences by the truncated GPIIb promoter, the GPIIb promoter was digested by HindIII-BamHI, treated with Klenow DNA polymerase to obtain blunt extremities and then introduced in BstEII-NcoI-opened pUT535 treated with Klenow DNA polymerase. The GPIIb- β -Gal vector was named: pUT535-GPIIb (Figure 2). The negative control plasmid

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(pUT535- δ CMV) was the pUT535 deleted of the CMV sequences by BstEII-NcoI digestion.

B. Tissue-specificity of the GPIIb promoter in hematopoietic cells

pUT535, pUT535 δ CMV and pUT535-GPIIb constructs were tested in two different cell lines: HEL and HeLa cells. The HEL cell line (Human Erythroleukemia Cells) is a classical cell line used to test megakaryocytic promoter expression. HeLa cells (Human cervix epitheloid carcinoma) are their classical negative control counterpart.

Materials and methods:

HEL cells were maintained in RPMI/10% FCS medium with 5% CO₂. β -Gal vectors (2 μ g DNA) were transfected in HEL cells (1×10^6 cells) using FUGENETM 6 (Boehringer Mannheim). After overnight incubation, the cells were harvested and placed in 10 ml of RPMI/1% BSA (Boehringer, Mannheim). PMA (Sigma) was added to the medium in one of the two assays (final concentration: 1nM). HeLa cells were maintained in DMEM-10% FCS with 5% CO₂. 90 mm Petri dishes with 5×10^6 cells were prepared the day before transfection. The cells were treated with 20 μ g DNA and 20 μ l LipofectinTM reagent (Gibco) for 5 hours. After this incubation time, the cells were washed and placed in DMEM-1% BSA with or without PMA 1nM. The cells were harvested 40 hours later, incubated with a lysis buffer, and the lysates were diluted to a final protein concentration of 1.5 mg/ml. The chemiluminescent β -Gal reporter Gene Assay kit (Boehringer Mannheim) was used as described by the manufacturer to quantify the β -galactosidase.

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Results:

In HeLa cells, β -Gal reporter gene was expressed only when the cells were transfected with a vector containing the CMV promoter, without and with PMA induction. No β -Gal expression was found with the construct directed by GPIIb promoter (Figure 3, panel A).

In HEL cells (Figure 3, panel B), CMV gave the highest β -Gal production (Table 1 below). However, GPIIb promoter led to the production of a significant amount of β -Gal with an increase in production after PMA treatment. When the β -Gal amount obtained with pUt535 in HEL cells was taken as 100%, the β -Gal amount with truncated GPIIb promoter was 13% in the cells without PMA treatment. In comparison, Uzan et al. (1991) found that the CAT activity with the GPIIb promoter corresponded to 15% of the CAT activity obtained with the RSV promoter control in the same cell line.

	β -Gal -PMA		β -Gal +PMA	
	U/ml	%	U/ml	%
CMV promoter	2959	100	22910	100
GPIIB promoter	376	13	662	3

Table 1: β -Gal activity in transiently transfected HEL cells

In conclusion, -597/+33 GPIIb promoter induced a tissue-specific expression of the reporter gene in the hematopoietic cell line since no β -Gal expression was detected in HeLa cells. In these transient transfections of HEL cells, β -Gal production obtained with this hematopoietic-specific promoter was low in comparison with the results measured with the CMV promoter. However, the results obtained in vitro are similar to the results published by Uzan et al. (1991).

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C. FIX transient expression in HEL cells

To test the possibility of producing FIX in hematopoietic cells, HEL cells were transiently transfected with FIX vectors bearing either CMV or GPIIb promoters, called CMV-FIX and GPIIb-FIX, respectively. The study was first done with the FIX(-41S) constructs and the results were confirmed with the FIX WT constructs.

1. Materials and methods

7.5×10^6 HEL cells were transfected with 20 μ g DNA using 20 μ l Superfect Transfection reagent (Qiagen). After 6 hours, the cells were harvested and placed in RPMI/1%BSA \pm PMA 1nM at a final concentration of 2.5×10^5 cells/ml. The supernatants were collected after 96 hours and the cells were numbered.

For the FIX WT constructs, pcDNA3-FIX WT and pcDNA3 WT.11 have been transfected in HEL cells (1×10^6 cells) using 6 μ g. Eugene 6 and 2 μ g DNA. Following overnight incubation, the cells were harvested and placed in 5 ml of RPMI/1% BSA/PMA 1nM for CMV-FIX transfections and in 2.5 ml of induction medium for the GPIIb-FIX transfections. Four days after transfection, the supernatants were recovered and the cells were numbered.

2. Results with FIX(-41S) constructs

When the cells were incubated without PMA, the FIX production obtained with the CMV-FIX construct was very low and not accurately detectable by ELISA (Asserachrom FIX, Stago, Asnières, France). The results presented in Figure 4 were obtained after induction with 1nM PMA. Under CMV promoter control, FIX secretion by CMV-FIX (-41S)-transfected HEL cells was low (408.66 pg/ml) and not significant. The best production was obtained with the cells transfected with FIX(-41S.11).

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The mean production was 2-fold higher than with FIX(-41S) cDNA and was significantly different from the result obtained with FIX(-41S) cDNA ($p < 0.05$). A similar result was obtained when adjusting with the cell number. Indeed, HEL transfected with FIX(-41S.I1) produced 4.5 ± 2.5 ng/ 1×10^6 cells and this result was significantly different from the result obtained with FIX(-41S)-transfected HEL cells (1.5 ± 0.9 ng/ 1×10^6 cells; $p < 0.01$).

Similar experiments were done with the GPIIb-FIX constructs, and in this case (2.5×10^5 cells/ml after transfection), the FIX production was too weak to be detectable in the supernatants. Microsep™ microconcentrators (Pall Gelman Sciences) with a 30Kd cut-off were used to concentrate the proteins. In the concentrated GPIIb-FIX(-41S.I1) supernatant obtained with, PMA-stimulated HEL cells, FIX protein was barely detectable. This result was in agreement with the results obtained with the β -Gal reporter gene (Table 1), confirming that the GPIIb potential was low in comparison with the CMV promoter.

3. Results with FIX WT constructs

Figure 5 presents the FIX production obtained with the FIX WT. These results confirm that:

- HEL cells are able to produce FIX after transient transfection.
- The best production levels were obtained with FIX WT.I1 constructs and were significantly different from the corresponding FIX WT results.
- The GPIIb promoter was less effective for FIX production than the CMV promoter in HEL cells. GPIIb-FIX WT.I1-transfected HEL cells produced about 30 percent of the FIX production obtained with the same cDNA under

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CMV promoter control ($257,71 \pm 85,38$ pg/ 1×10^6 cells versus $881,28 \pm 288,32$ pg/ 1×10^6 cells).

D. FIX expression in stably transfected HEL cells

As mentioned above, in the transient expression system, a low FIX production was obtained. Therefore, cells stably expressing FIX were generated.

1. Materials and methods

Cells: HEL cells (1×10^6 cells) were transfected with 2 μ g of PvuI linearized plasmid during 5 hours using FUGENETM 6 transfection system (Boehringer, Mannheim). After incubation, the cells were harvested and placed in fresh medium supplemented with 0.6 mg/ml G418.

To compare FIX production, the G418-resistant cells were placed in RPMI/1% BSA supplemented with Vitamin K 1ng/ml \pm PMA 1nM (2.5×10^5 cells/mL). After 1, 2, 3 or 4 days of incubation, the cells were numbered and the supernatants were frozen. FIX concentrations were measured using a FIX ELISA kit (Asserachrom FIX, Stago, Asnières, France).

Immunoblotting analysis: Supernatants (1 ml) were diluted fourfold in an equilibration buffer (Hepes 10 mM, KCl 100 mM, MgCl₂ 2 mM, Triton x100 0.1%). Fifty microliters of anti-human factor IX antibody bound to DEAE A50 Sephadex kindly provided by CenteonTM (USA) were added to the supernatant solutions and incubated 1 hr at 4°C. The DEAE was then collected by centrifugation (2 min at 2500 rpm) and washed three times with the equilibration buffer. Factor IX was eluted by Laemmli buffer (Laemmli, 1970). Samples containing Factor IX were subjected to electrophoresis on SDS-PAGE/10% polyacrylamide gel and semidry blotted onto HybondTM C Pure membrane (Amersham). The blots were blocked with TBS-(0.15

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M NaCl, 10 mM Tris-HCl, pH 7.5) Tween (0.1%) for 1 hr at room temperature and then incubated with 1:3,000 dilution of a polyclonal rabbit anti-human FIX antibody (Dako™). The membrane was washed 3 times in TBS-T and then incubated with a 1:10,000 dilution of a peroxidase-labeled anti-rabbit antibody (Biorad) for 30 min. After washing 3 times, a chemiluminescent signal was detected by autoradiography using the ECL System (Amersham™).

Procoagulant activity: Factor IX activity was determined by a one-stage clotting assay. Fifty microliters of control plasma were diluted (1/10) in imidazole buffer (Diagnostica Stago, Asnières, France). Fifty microliters of diluted supernatant were added to 50 µl of deficient Factor IX plasma (Immuno, Illkirch, France) and to 50 µl of Silimat® cephalin (Bio-merieux™, Marcy l'Etoile, France). After 4 min incubation at 37°C, clotting was initiated by addition of 50 µl of 50 mM CaCl₂ buffer. Coagulation times were measured in a STA Compact® coagulometer (Diagnostica Stago™ Asnières, France) and Factor IX activity was determined from a log-log standard curve.

2. Daily FIX production of FIX(-41S.I1) expressing HEL cells

2.1 CMV-FIX(-41S.I1) transfected HEL cells

No FIX was detected in the supernatants of pcDNA3-transfected HEL cells (Figure 6). After 4 days, 11,65 ng/ml of FIX were detected in the conditioned medium of the CMV-FIX(-41S.I1) transfected cells. Following PMA induction, FIX production (259,87 ng/ml) was 23-fold higher than the production obtained without PMA and was 250-fold higher than the result obtained with transient transfections.

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2.2. GPIIb-FIX(-41S.I1) transfected HEL cells

When HEL cells were transfected with pcDNA3-GPIIb vector, no FIX was detected in the conditioned medium. With the GPIIb-FIX(-41S.I1) HEL cells, FIX increased regularly during the 4 days of incubation (Figure 7). After 4 days of culture, GPIIb-FIX(-41S.I1) pools produced 35.0 ± 7.8 ng/ml of FIX in the supernatant. Following PMA induction, the FIX production reached 165.0 ± 32.52 ng/ml.

2.3. Procoagulant activity

The FIX procoagulant activity was directly correlated with the FIX antigen in the supernatants from HEL cells (Figure 8). The specific activity of the FIX produced by GPIIb-FIX(-41S.I1)-expressing HEL cells (culture medium: RPMI/1% BSA) was 0.34 ± 0.07 ng/ml.

2.4. FIX immunoprecipitations

The supernatants of transfected HEL cells were immunoprecipitated and Western blotted to verify the molecular weight of the secreted protein. Purified recombinant Factor IX (Mononine™) was used as control. In the supernatants of pcDNA3-GPIIb expressing cells, no FIX was detected. In the GPIIb-FIX(-41S.I1) HEL cell supernatants without or with PMA induction, one specific band was detected with an approximative molecular weight of 60 kDa. This protein migrated at the same level as immunopurified FIX (Mononine™), as presented in Figure 9 (the construct pcDNA3-GPIIb-FIX(-46;I1) was obtained with another FIXcDNA comprising a different modified 5'extremity).

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E. Conclusion

The above-mentioned results show that the hematopoietic cell line HEL can produce and secrete fully active FIX. After transient transfections, the best production was obtained with CMV-FIX (-41S.11) HEL cells in comparison with the CMV-FIX(-41S) transfected HEL cells, which demonstrated the expression-increasing effect of the Intron 1 in this hematopoietic cell line. Using the GPIIb promoter, the FIX production was tissue-specific. The FIX production obtained in HEL cells stably transfected with GPIIb-FIX were equivalent to the productions obtained with the CMV-FIX transfected cells and indicated that the effectiveness of the GPIIb promoter was similar to the effectiveness of the CMV promoter in hematopoietic cell lines. The secreted FIX protein has a molecular weight of about 60 kDa, similar to the FIX contained in MononineTM and then seems to undergo a normal maturation process. Finally, the FIX coagulant activity using a one-stage assay corresponds to the FIX antigen, indicating that the recombinant molecule is fully active. These results obtained with HEL cells reinforce the hypothesis that FIX protein can be produced by CD34+ hematopoietic stem cells.

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CENTEON PHARMA GMBH
ANR 8177007

1999/Z004 – Ma 1204 – C 36
Dr. Lp / vm

EPO - Munich
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27. April 1999

Claims:

1. DNA-construct for the tissue-specific expression of a blood coagulation factor comprising a DNA coding for an amino acid sequence of a blood coagulation factor and a DNA coding for a promoter which is specific for the expression in hematopoietic cells.
2. DNA-construct as claimed in claim 1, wherein as promoter the DNA coding for the human platelet glycoprotein IIb (GPIIb) is inserted.
3. DNA-construct as claimed in claims 1 and 2 wherein the DNA coding for the amino acid sequence of an blood coagulation factor is the cDNA coding for Factor IX.
4. DNA-construct as claimed in claims 1 to 3, wherein the first truncated intron (Intron 1) of the human FIX gene has been inserted additionally into the FIX-cDNA.
5. Process for the production of Factor IX wherein the Factor IX is expressed in a hematopoietic cell line into which the DNA-construct of claims 1 to 4 has been transfected.
6. Process as claimed in claim 5, wherein the production of Factor IX is stimulated by an inducer.
7. Process as claimed in claim 6, wherein the production of Factor IX is stimulated by phorbol-12-myristate-13-acetate (PMA).

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CENTEON PHARMA GMBH
ANR 8177007

1999/Z004 - Ma 1204 - C 36
Dr. Lp / vm

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27. April 1999

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+33

GTGGAAGAAG GAAG

674

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<212> DNA

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- 3 -

<213> Homo sapiens

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+33

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BamHI

GTGGTGAAT TCTGCAGATA TCCAGCACAG TGGCGGCCGC TCGAGCTCAG AGGGCCCGTT
120

NotI XhoI

TAAACCC

127

Fig.1

Cloning of GPIIb promoter in pcDNA3 vector

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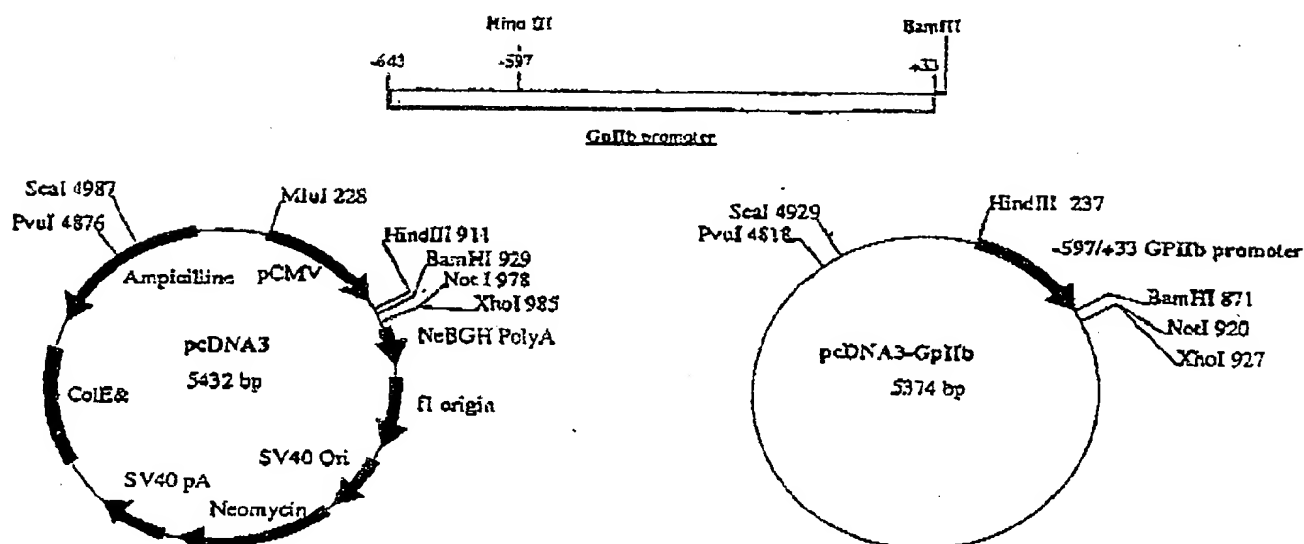


Fig.2

β -Galactosidase reporter gene vectors

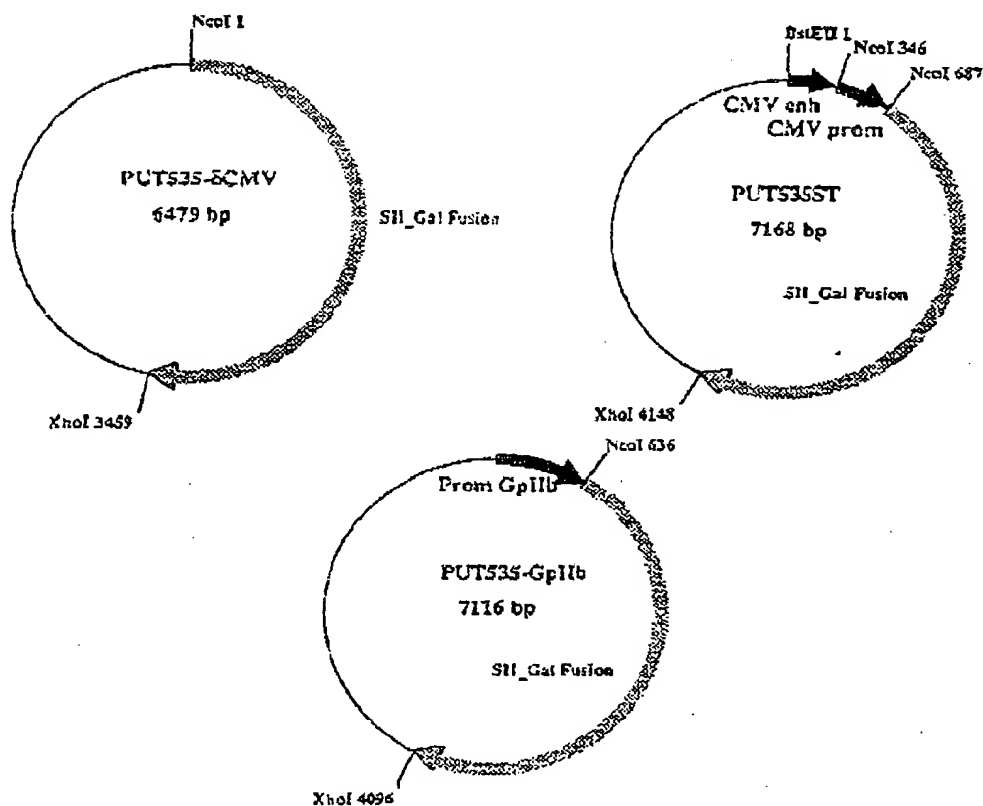


Fig.3

β -Gal transient expression directed by GPIIb in HeLa cells
(panel A) and HEL cells (panel B)

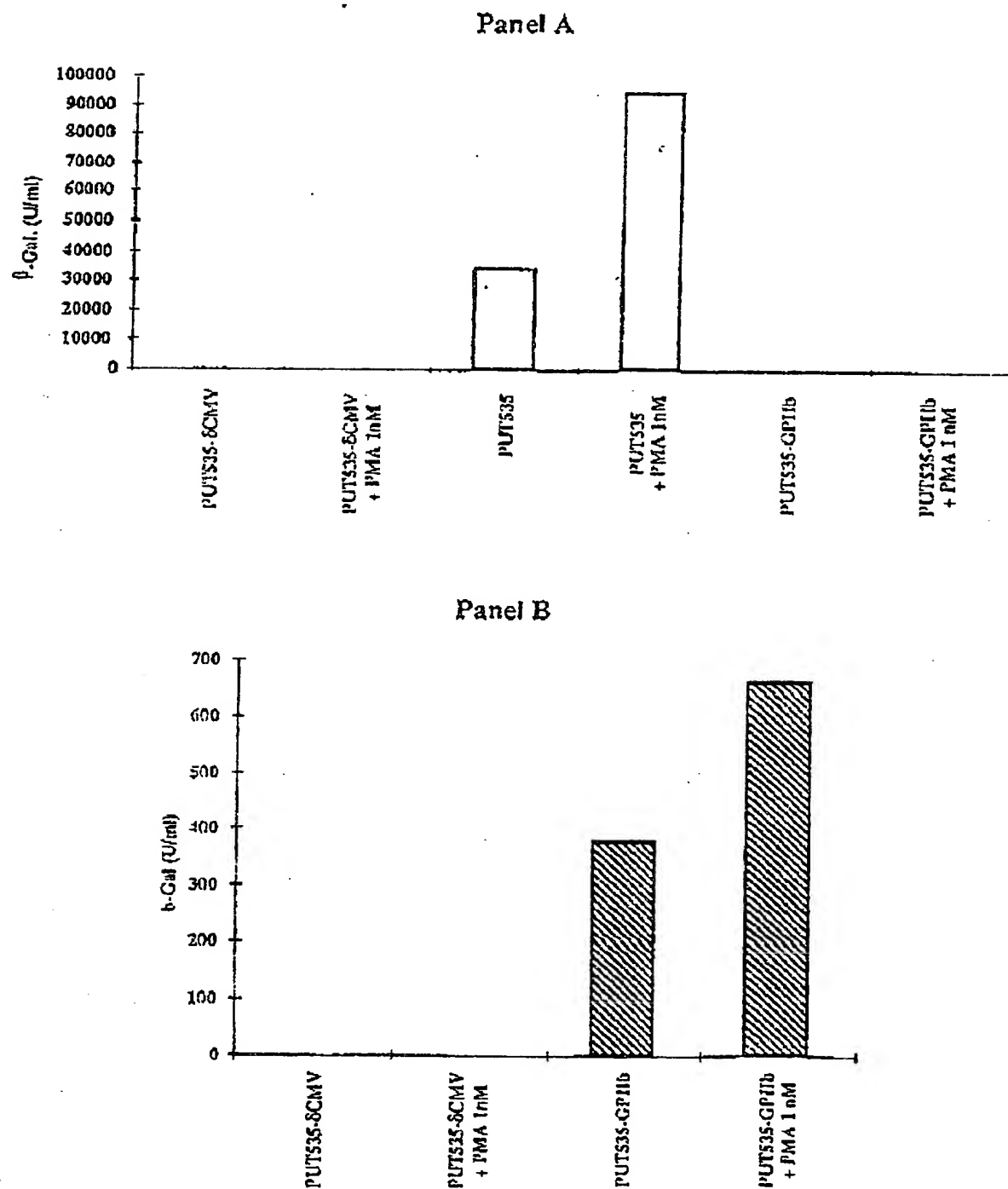
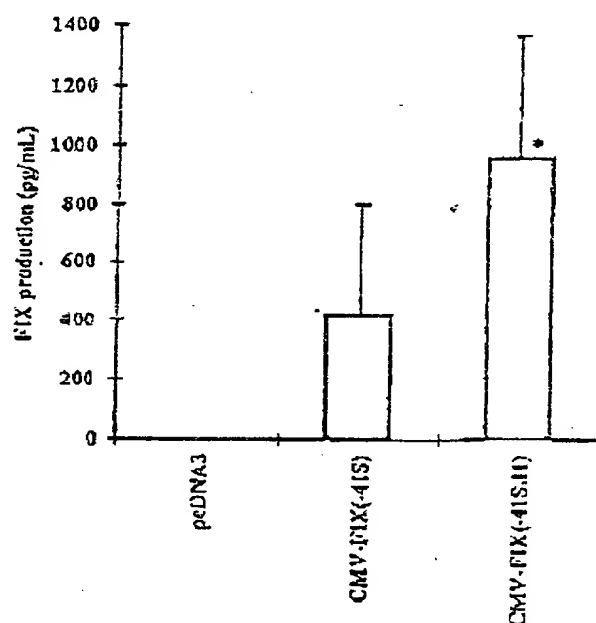


Fig.4**Transient FIX production under CMV promoter control in HEL cells
after PMA 1nM induction**

(pcDNA3 was used as reference for statistics (*represents $p < 0.05$))

**Fig.5****Transient FIX WT production under CMV or GPIIb promoter control in
HEL cells after PMA 1nM induction**

pcDNA3 and pcDNA3-GPIIb respectively were used as reference for statistics

(** represents $p < 0.01$)

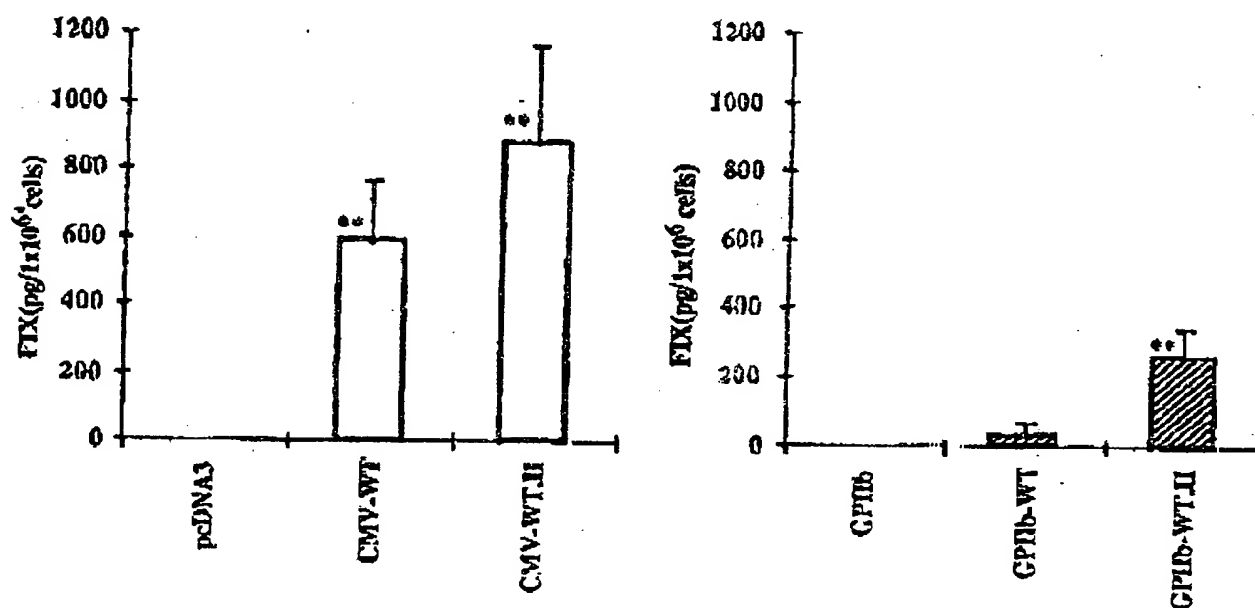


Fig.6

Daily FIX production using CMV promoter in stably transfected HEL cells

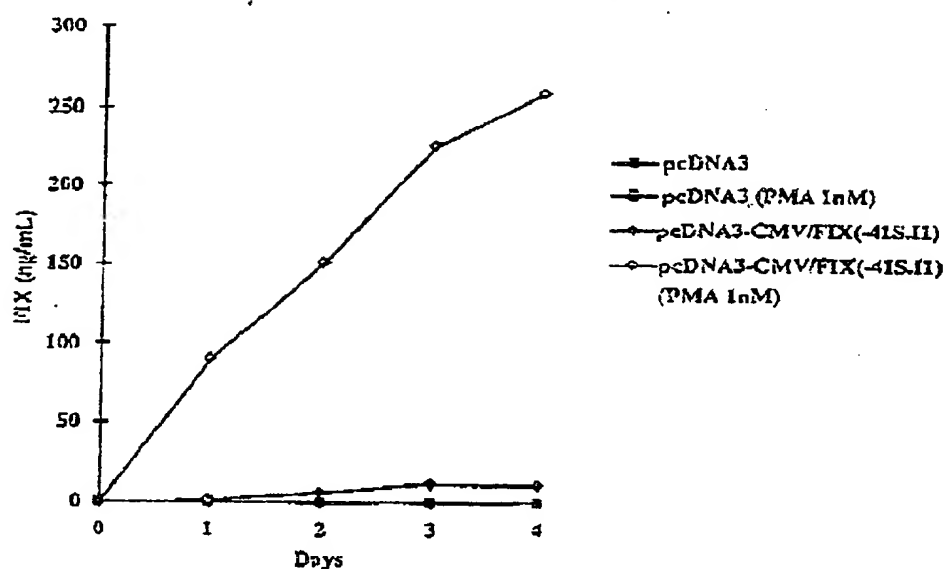


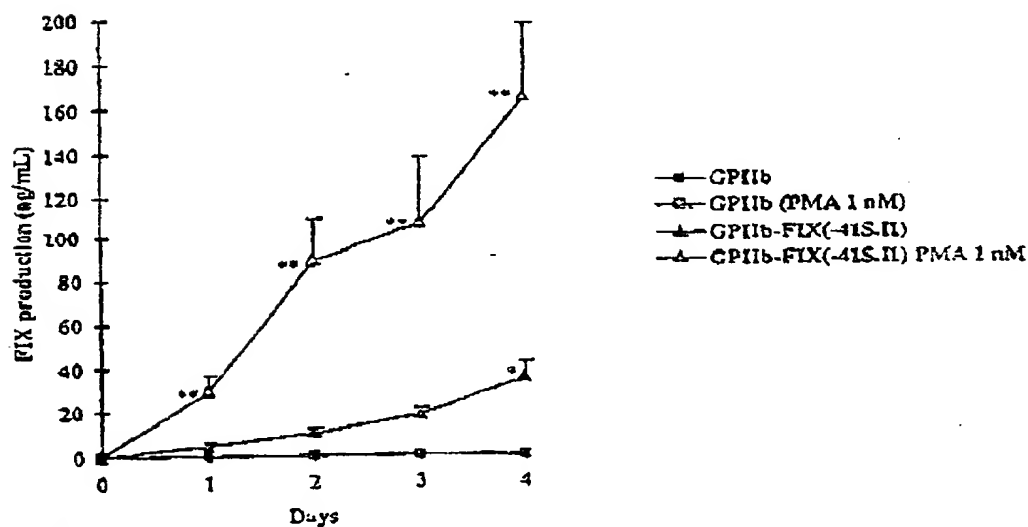
Fig.7

FIX production under GPIIb promoter in stably transfected

HEL cells with or without PMA induction

pcDNA3-GPIIb construct was used as reference for statistics

(* represents $p < 0.05$; ** represents $p < 0.01$)



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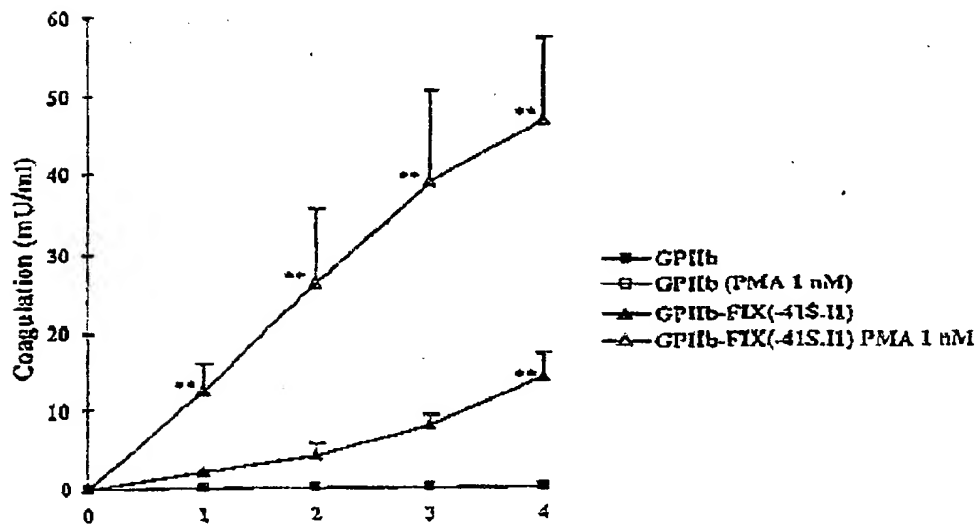
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Fig.8

Procoagulant FIX activity in HEL cells supernatants

PcDNA3-GPIIb construct was used as reference for statistics

(* represents $p < 0,05$; ** represents $p < 0,01$)

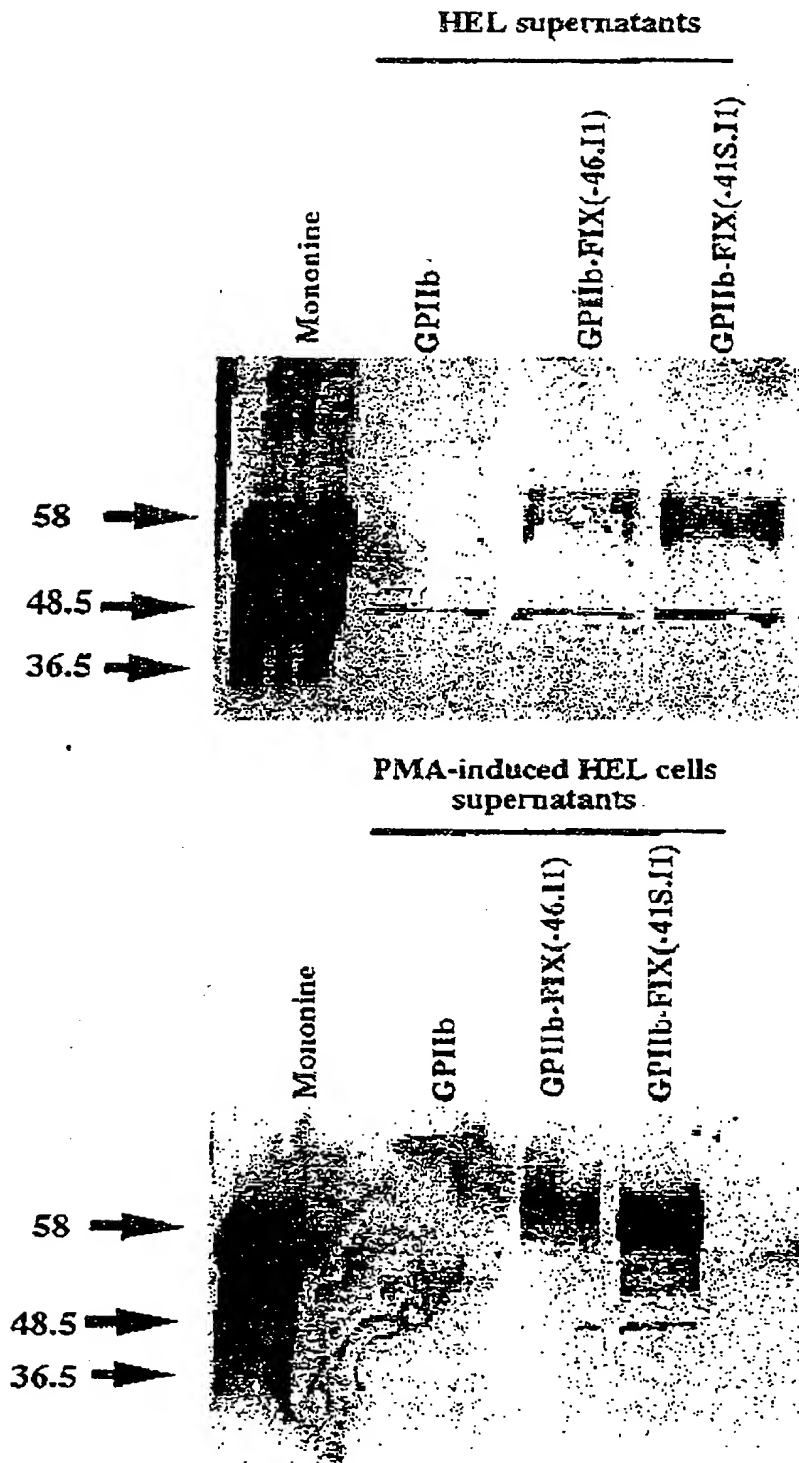
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Fig.9

**Immunoblot analysis after immunoprecipitation of FIX
in HEL cells supernatants**



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CENTEON PHARMA GMBH
ANR 8177007

1999/Z004 - Ma 1204 - C 36
Dr. Lp / vm

EPO - Munich
21
27. April 1999

Abstract:

DNA-construct for the tissue-specific expression of a blood coagulation factor

A DNA-construct has been disclosed which is suitable for a tissue-specific expression of a blood coagulation factor like Factor IX, which comprises a DNA coding for an amino acid sequence of a blood coagulation factor and a DNA coding for a promoter which is specific for the expression in hematopoietic cells.

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